CXCR2 INHIBITORS AND PMN ADHESION AND T-CELL CHEMOTAXIS

Field of The Invention

The present invention relates to the novel use of CXCR2 inhibitors for the treatment of diseases mediated thereby.

Background of the Invention

The recruitment of inflammatory cells into sites of tissue damage is a normal physiological response designed to fight infection, remove damaged cells, and stimulate healing. However, the excessive recruitment of such cells often exacerbates tissue damage, slows healing and in some cases leads to host death. Therefore, inhibition of inflammatory cell recruitment may be an appropriate therapeutic strategy in a number of inflammatory diseases, such as reperfusion injury, arthritis, asthma atherosclerosis and inflammatory bowel disease.

Summary of the Invention

The present invention is to a method of inhibiting, or blocking, the binding of human neutrophils to activated endothelial cells in a patient in need thereof, which method comprises administering to said patient an effective amount of a compound which binds the CXCR2 receptor.

Preferably, the compound will also bind to other chemokine receptors, such as the CXCR1 receptor.

Another aspect of the present invention is a method of inhibiting or blocking T-cell mediated chemotaxis in a patient in need thereof, which method comprises adminstering to said patient an effective amount of a compound which binds the CXCR2 receptor.

Brief Description of the Drawings

Figure 1 shows a control experiment. in which Human Umbilical Cord EndothelialCells (HUVEC) are treated with IL-1β and IL-8 in the absence of CXCR2 antagonist. IL-1β and IL-8 stimulate neutrophils to roll along the endothelial cell surface and to firmly adhere to these cells.

Figure 2 demonstrates that administration of compound 1, N-[2-Hydroxy-4-cyanophenyl]-N'-[2-bromophenyl] urea blocks the firm adhesion of neutrophils to the cell surface of HUVEC but does not interfere with rolling

of PMNs on HUVEC.

Figure 3 shows the dose dependent inhibition of PMNs binding to activated HUVEC by Compound 1. Each point represents the percent of cells (compared with control) which have bound to the endothelial cells at the 4 min time mark at each of the different concentrations of Compound 1.

Figure 4 shows the dose dependent inhibition of T-cell mediated migration by compound II, N-(2-Hydroxy-4-nitrophenyl]-N'-(2-bromophenyl)urea when T-cells were stimulated to migrate to IL-8 or Groα but not to a control chemokine MCP-1.

Detailed Description of the Invention

The recruitment of neutrophils from post-capillary venules depends initially upon the rolling of neutrophils via the interaction of neutrophils expressed sLex with endothelial cells expressed E-selectin. This is followed by attachment through the up-regulation of the adhesion molecules CD11b/CD18 (Mac-1), and diapedesis via a heptotactic gradient of IL-8 (Rot, et al., *J. Leukoc. Biol.* **59**, 39-44 (1996)). The mechanism for PMN attachment to endothelial cells is not completely understood, but may well involve the up-regulation of CD11b/CD18 on neutrophils (Detmers. et al., *J. Exp. Med.* **171**, 1155-1162 (1990)). T-cells also respond to the chemokines IL-8 and Groα and migrate to these two factors in a similar manner as neutrophils. The recruitment of T-cells to sites of antigen presentation such as can be found in arthritic joints of patients suffering from rheumatoid arthritis. is thought to be essential for the continuation of the inflammatory process.

IL-8 and Groα are members of the super family of proinflammatory proteins known as chemokines, which are approximately 8 kD in size. In human neutrophils and T-cells, IL-8 binds with similar affinity to two distinct 7TMRs, CXCR1 (Holmes et al., *Science* **253**, 1278-1280 (1991)) and CXCR2 (Murphy, et al., *Science* **253**, 1280-1283 (1991)), whereas closely related chemokines containing a common amino-terminal Glu^4-Leu^5-Arg^6 (ELR) amino acid sequence, including GRO-α, NAP-2 and ENA-78, bind only to CXCR2 (Hebert et al., *J. Biol. Chem.* **266**, 18989-18994 (1991)). Both CXCR1 and CXCR2 are present on the surface of human neutrophils and a subset of T-cells (see Holmes, et al., *Supra*; Murphy et al., *Supra*; Chuntharapai et al., *J. Immunol.* **153**, 5682-5688 (1994) and Xu et al., *J. Leukoc. Biol.* **57**, 335-342 (1995)). In human neutrophils it is unclear whether attachment to

endothelial cells is mediated by one or both receptors. In addition, it is unclear which of these two receptors expressed on human T-cells are responsible for mediating T-cell chemotaxis.

One aspect of the present invention therefore, is to a method of inhibiting, or blocking, the binding or attachment, of human neutrophils to activated endothelial cells in a patient in need thereof, which method comprises administering to said patient an effective amount of a compound which binds the CXCR2 receptor. Another aspect of the present invention, is a method of inhibiting or blocking T-cell mediated movement or chemotaxis by administering to a patient in need of this treatment a compound which binds the CXCR2 receptor. Preferably, the compound will also bind to other chemokine receptors, such as the CXCR1 receptor.

Suitable CXCR2 inhibitors which are useful in the present invention include, but are not limited to those compounds disclosed in US Patent No. 5,684,032 ; WO 96/25157 ; US Patent No. 5,780,483 ; WO 97/35572 ; WO 97/49286; WO 97/49399; WO 97/49680; WO 97/49287; WO 98/07418 ; WO 97/49400 ; WO 98/05329 ; WO 98/05317 ; WO 98/05328 ; WO 98/06398 ; WO 98/06397 ; WO 98/06399 ; WO 98/06262 ; WO 98/06701 ; WO 98/ 32439 ; and WO 98/ 32438 ; Attorney Docket No.: P50708, PCT US98/ , filed ____; and Attorney Docket No.: P50709, PCT US98/----, filed ---/ / 98

BIOLOGICAL METHODS

HUVEC preparation and activation

HUVECs at passage 2 or 3 were grown for 24 to 48 hours in glass capillary tubes until confluent in EGM culture medium (Clonetics) containing 10% FBS. The endothelial cells were then activated just prior to the assay for 1hour with 10ng/ml IL-1, washed with EGM and then incubated with IL-8 (100ng/ml) or PAF (10nM) for 3 hrs at 37°.

Neutrophil isolation

Neutrophils were isolated from human peripheral blood. Briefly, blood was collected into citrate (ACD) anti-coagulant tubes (Becton Dickinson), diluted 1:2 at room temperature in sterile buffered HBSS w/o Ca^{++} & Mg^{++} (pH 7.0) with HEPES (20mM) (Fisher Scientific) then adding 5% Dextran T500 (Pharmacia Biotech) to the blood for a 1% final concentration. The blood/HBSS was then incubated at 4 °C for 45 minutes to allow the RBCs to settle out of suspension. The remaining RBCs in suspension were lysed in cold water for 10-15 seconds and then the leukocyte

suspension was mixed 10 part HBSS with 1 part water, spun down and resuspended at a concentration of $6x10^6$ cells/ml in HBSS w/o Ca^{++} & Mg^{++} (pH 7.0). The cell suspension was then underlayed with Histopaque 1077 and Histopaque 1117 (Sigma), and centrifuged at 2,300 RPM for 30 min at room temperature. neutrophils were collected from the Histopaque 1117/1077 interface. The cells were then resuspended at a concentration of $6x10^6$ cells/ml in HBSS Then, the cells were treated with Compound 1 (or not) and incubated on ice for 15-20 minutes. Just before adding the neutrophils for the loop assay they were diluted to $3x10^6$ cells/ml in DMEM with HEPES at 20mM.

T-cell Isolation

Peripheral blood mononuclear cells were harvested from individuals that had been sensitized with Tetanus Toxin 7 days prior to the blood draw. PBLs were separated over Ficol, and washed in PBS (x2) prior to adding the cells (1 x 106 cells/ml, RPMI1640 + 5% Autologous human serum) to T75 flasks with 5 ng of Tetanus Toxin. After 5 days of culture non-adherent cells were removed and washed 2x in PBS. These cells were then used for the chemotaxis assay. The chemotaxis assay was carried out as previously described (White et. al. J. Biol. Chem 273:10095 (1998)). After chemotaxis for 5 hrs in which chemokine was placed in the bottom chamber along with a suitable concentration of compound II, N-(2-Hydroxy-4-nitrophenyl]-N'-(2-bromophenyl)urea. The migrated cells were enumerated by staining the separation membrane DiffQuick and counting the number of T-cells which had migrated to the stimulus.

Effect of Compound 1 on selectin/integrin mediated neutrophil rolling and arrest on IL-8 pretreated, IL-1 activated HUVEC

The analysis of Compound 1: N-[2-Hydroxy-4-cyanophenyl]-N'-[2-bromophenyl] urea was performed in triplicate in DMEM medium containing 1% human serum buffered with 20mM HEPES. Untreated human neutrophils were isolated and handled as previously indicated and infused into the shear system loop for recirculation through the capillary tube lined with confluent cultures of HUVEC treated with IL-1 and IL-8 or PAF as indicated above. Neutrophils were pretreated (or not) with compound 1 antagonist at 300, 150, 50 and 10nM for 15-20 minutes before infusion into the shear assay.

General in vitro shear assay

After activation, the HUVEC containing capillary tubes were connected to the assay tubing to form a closed loop in which medium and cells could be recirculated; the tube was then mounted on the inverted microscope stage. Using a variable speed peristaltic pump, flow was regulated to simulate in vivo shear conditions (1.8 - 2 dynes/cm^2) (Bargatze, et al., J. Immunol. **152**:581 (1994)). Isolated human neutrophils were infused into the system at a 3x10^6 cell/ml in sterile HEPES buffered (20mM) HBSS (pH 7.0) plus 1% human serum. Rolling was established and the adhesive interactions were continuously monitored for the duration of the experiment while being videotaped for off-line analysis.

Analyses of neutrophil rolling

The number of neutrophils interacting, both binding and rolling, with activated HUVECs was quantified at 1 min intervals using NIH IMAGE software, Montana ImmunoTech Inc. macros and an Apple Computer PowerMac 7100-66. Interacting neutrophils were quantified, within 350 µm (horizontal) by 250 µm (vertical) video-microscopic fields.

Results

Data from three replicate experiments show that Compound 1, after a 15 - 20 min preincubation with neutrophils inhibits IL-8 enhanced adhesion to IL-1b activated HUVECs over a 8 minute time interval. Compound 1 did not inhibit rolling of neutrophils over the HUVEC cell surface (Figures 1 and 2). In addition, Compound 1 dose dependently inhibited neutrophil binding to HUVEC (Fig 3) with an IC50 = 20 nM. When PAF (Platelet Activating Factor) was substituted for IL-8 in the shear assay, Compound 1 at a concentration of 300 nM failed to inhibit binding of PMN's to the HUVEC cells, thus indicating that Compound 1 is a specific inhibitor of IL-8 induced function. Data also indicated that compound II was capable of inhibiting both IL-8 and Groα mediated T-cell chemotaxis but was not able to inhibit a related chemokine MCP-1 (Figure 4).

All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as if each individual publication were specifically and individually indicated to be incorporated by reference herein as though fully set forth.

The above description fully discloses the invention including preferred embodiments thereof. Modifications and improvements of the embodiments specifically disclosed herein are within the scope of the following claims. Without further elaboration, it is believed that one skilled in the ~~are~~ art can, using the preceding description, utilize the present invention to its fullest extent. Therefore, the Examples herein are to be construed as merely illustrative and not a limitation of the scope of the present invention in any way. The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows.